Immunity

Supplemental Information

Interleukin-2 Activity can be Fine-Tuned with Engineered Receptor Signaling Clamps

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Table S1, related to Figure 4. Genes that are up- or down-regulated by IL-2, H9, H9-RET, and H9-RETR as compared to unstimulated control cells. Note that this is provided as a separate Excel file.

Figure S1, related to Figure 2. Effects of IL-2 variants on pERK. (A and **B)** Dose-response curves of phospho-ERK1/ERK2 protein on $CD25^+$ (**A**) and $CD25^-$ (**B**) YT1 human NK-like cells. **(C)** Comparison of IL-2Rβ and IL-2Rγ expression on freshly isolated (upper panels) or pre-activated (lower panels) human $CD8^+$ T cells. Data are representative of at least two experiments per panel. Error bars in **(A)** represent S.E.M. of triplicates.

Figure S2, related to Figure 5. Effect of H9-RET and H9-RETR on CD25 expression, IL-2- and IL-15-induced proliferation, and TCR-induced proliferation of $CD8^+$ T cells, and the differentiation of Th1, Th17, Th9, and iTreg cells.
(A) H9-RET and H9-RETR inhibited IL-2-induced CD25 expression on pre-activated $CD8^+$ T cells. The numbers represent the percentage of cells in the corresponding quadrants. Data are representative of three independent experiments.
(B) Inhibition of IL-2- and IL-15-induced proliferation by H9-RET and H9-RETR. Freshly isolated $CD8^+$ T cell were CFSE-labeled and stimulated with IL-2 or IL-15 (1 μg/ml) in the presence or absence of 1 μg/ml H9-RET or H9-RETR, and CFSE dilution assessed. Data are representative of three independent experiments.
(C) Both H9-RET and H9-RETR inhibit TCR-induced proliferation of freshly isolated $CD8^+$ T cells. Cells were labeled with CFSE, stimulated with plate bound anti-CD3 (2

μg/ml) + soluble anti-CD28 (1 μg/ml) for 4 days with or without 1 μg/ml of H9-RET or H9-RETR, and CFSE dilution assessed by flow cytometry.

(D) 1 μg/ml of either H9-RET or H9-RETR inhibited TCR-induced CD25 expression on peripheral blood $CD8^+$ T cells stimulated for 4 days with 2 μg/ml anti-CD3 + 1 μg/ml anti-CD28. Data are representative of three independent experiments.

(**E**) H9-RET and H9-RETR block Th1, Th9, and Treg differentiation but promote Th17 differentiation. Cells were differentiated under various T-helper polarizing conditions in the absence or presence of H9-RET or H9-RETR. Data are representative of at least two independent experiments for each type of cell.

Figure S3, related to Figure 6. Relative potency *in vivo* of H9-RETR-Fc4 versus an anti-mouse-IL-2Rα blocking antibody (clone PC61).

H9-RETR-Fc4 was more effective in blocking IL-2-induced pSTAT5 signaling than PC61 *in vivo*. Left, C57BL/6 mice were injected i.p. with PBS or 1 μg IL-2. Right, C57BL/6 mice were injected with 100 μg of control rat IgG1, PC61, or H9-RETR-Fc4 60 min prior to injection of 1 μg IL-2. pSTAT5 was measured 30 min later in splenic $CD4^+CD25^+FoxP3^+$ T cells. MFIs are indicated. Data are representative of two independent experiments.

Figure S1

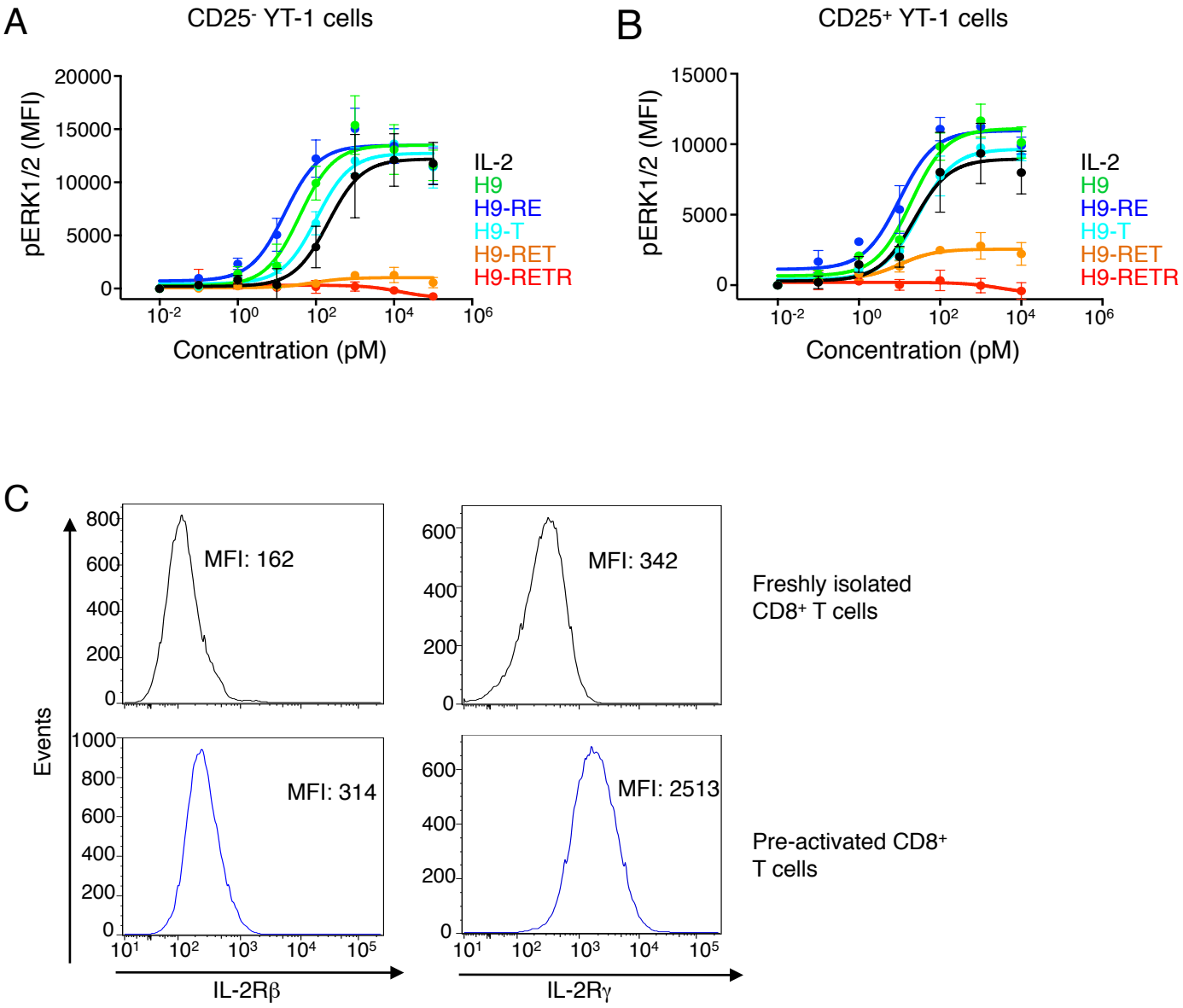

A
CD25- YT-1 cells
pERK1/2 (MFI)
20000
15000
10000
5000
0
10-2
100
102
104
106
Concentration (pM)
IL-2
H9
H9-RE
H9-T
H9-RET
H9-RETR
B
CD25+ YT-1 cells
pERK1/2 (MFI)
15000
10000
5000
0
10-2
100
102
104
106
Concentration (pM)
IL-2
H9
H9-RE
H9-T
H9-RET
H9-RETR
C
Events
MFI: 162
MFI: 342
MFI: 314
MFI: 2513
IL-2Rβ
IL-2Rγ
Freshly isolated
CD8+ T cells
Pre-activated CD8+
T cells

Figure S2

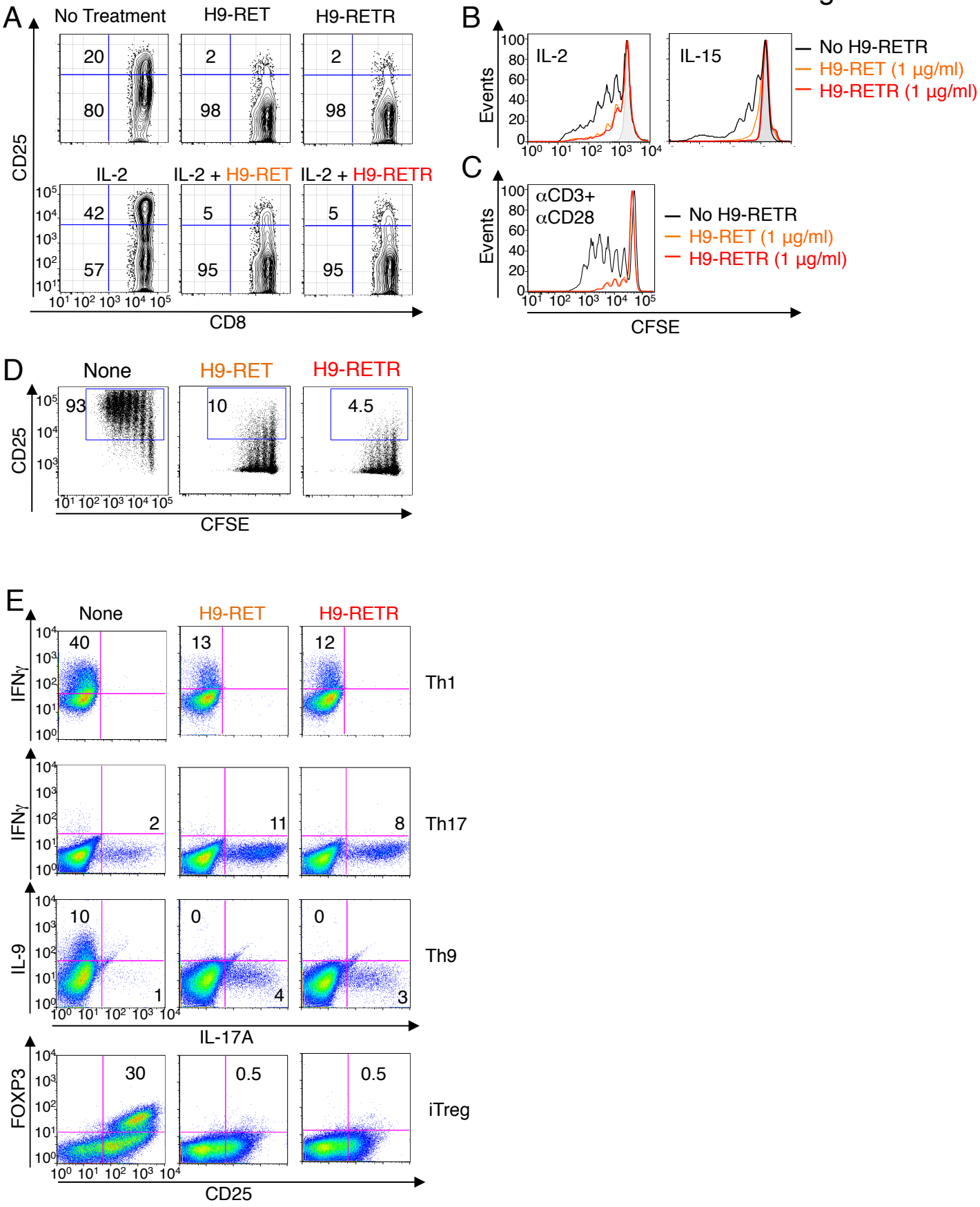
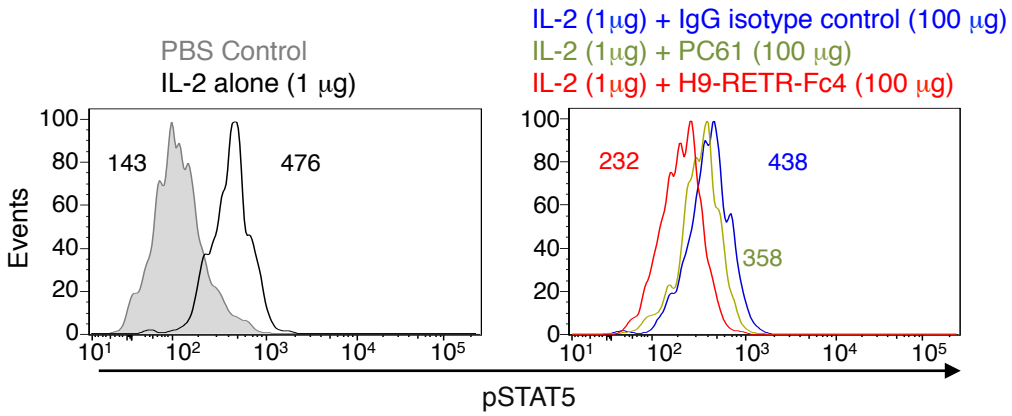

A
No Treatment
H9-RET
H9-RETR
20
80
2
98
2
98
IL-2
IL-2 + H9-RET
IL-2 + H9-RETR
42
57
5
95
5
95
CD25
CD8
B
IL-2
IL-15
Events
No H9-RETR
H9-RET (1 µg/ml)
H9-RETR (1 µg/ml)
C
αCD3+
αCD28
Events
No H9-RETR
H9-RET (1 µg/ml)
H9-RETR (1 µg/ml)
CFSE
D
None
H9-RET
H9-RETR
93
10
4.5
CD25
CFSE
E
None
H9-RET
H9-RETR
40
13
12
Th1
IFNγ
2
11
8
Th17
IFNγ
10
1
0
4
0
3
Th9
IL-9
IL-17A
30
0.5
0.5
iTreg
FOXP3
CD25

Figure S3


PBS Control
IL-2 alone (1 μg)
IL-2 (1μg) + IgG isotype control (100 μg)
IL-2 (1μg) + PC61 (100 μg)
IL-2 (1μg) + H9-RETR-Fc4 (100 μg)
Events
143
476
232
438
358
pSTAT5

Supplemental Experimental Procedures

Flow Cytometric Analysis of Intracellular Phospho-STAT5 and Phospho-ERK1-ERK2 Approximately 2 x 10^5 YT or $CD25^+$ YT-1 cells were plated in wells of a 96-well plate, washed with flow cytometry buffer, and re-suspended in flow cytometry buffer containing serial dilutions of IL-2, H9, H9-RET, or H9-RETR. Cells were stimulated for 20 min at 37ºC, fixed by addition of formaldehyde to 1.5%, incubated for 10 min at room temperature, and permeabilized with 100% ice-cold methanol for 30 min at 4ºC to allow for detection of intracellular signal effectors. The fixed and permeabilized cells were washed twice with flow cytometry buffer and incubated with Alexa488-conjugated anti-STAT5 pY694 (BD Biosciences) or Alexa488-conjugated anti-ERK1-ERK2 pT202/pY204 (BD Biosciences) diluted in flow cytometry buffer for 2 hr at room temperature. Cells were then washed twice in flow cytometry buffer and mean fluorescence intensity (MFI) was quantified on an Accuri C6 flow cytometer (BD Biosciences). Dose-response curves were generated, and EC_{50} and E_{max} values were calculated using the GraphPad Prism data analysis software after subtracting the MFI of unstimulated cells and normalizing to the maximum signal intensity induced by cytokine stimulation.

Intracellular Staining of pSTAT5 and pS6-Ribosomal Protein in primary human CD8 cells.

Dose-response experiments on primary human $CD8^+$ T cells were performed as described (Ring et al., 2012); in brief, cells were treated with serial dilutions of IL-2, H9, H9-RET,

H9-RETR, then fixed with Phosflow Fix Buffer I at room temperature for 10 min (BD Biosciences), and washed once with flow cytometry buffer. Cells were then permeabilized by slowly adding cold BD PhosflowTM Perm Buffer III and incubated for 30 min on ice. Cells were washed and stained with PE-conjugated anti-STAT5 pY694 (BD Biosciences) or APC-conjugated anti-phospho-S6 ribosomal protein (Ser235/236) (clone D57.2.2E) at room temperature for 30 min in the dark, washed again with flow cytometry buffer, and data acquired on a FACSCanto II flow cytometer (BD Biosciences) and analyzed with FlowJo (Tree Star).

Inhibition of IL-2-induced pSTAT5

Freshly isolated $CD8^+$ T cells and $CD8^+$ T cells pre-activated with anti-CD3 + anti-CD28 were stimulated with IL-2 in the absence or presence of H9-RET or H9-RETR, and pSTAT5 assessed. Cells were incubated with or without anti-Tac or Mikβ1, or H9-RETR-Fc4 for 1 hr, stimulated with a range of doses of IL-2 or IL-15 for 30 min, and pSTAT5 measured by flow cytometry. For NK cell experiments, freshly isolated human NK cells (NK Cell Isolation Kit II, Miltenyi Biotech) were stimulated with serial dilutions of IL-15 in the presence or absence of the indicated IL-2 variant, and pSTAT5 assessed.

Western Blotting

Cells stimulated with or without cytokines were lysed in RIPA buffer containing 1% IGEPAL CA-630 (Sigma). Equal amounts of lysates were resolved on 4 to 12% Bis-Tris NuPAGE gels (Invitrogen), transferred to membranes, and the membranes incubated for 1 h at room temperature with antibodies to pSTAT5(Y694) (Cell Signaling Technology,

Inc., Beverly, MA) or STAT5 (BD Transduction Laboratories, San Diego, CA). Bound antibodies were detected with goat anti-rabbit- IgG (H+L)-HRP conjugate (1:5,000 dilution) and with goat anti-mouse IgG (H+L)-HRP conjugate (1:10,000 dilution) (Biorad). Immunoblots were visualized using enhanced chemiluminescence (ECL, GE healthcare). In some experiments, membranes were reused after incubating in stripping buffer (Millipore) for 15 min at room temperature.

Thymidine incorporation assay

Total human $CD8^+$ T cells were isolated from PBMCs and added to anti-CD3 (2 μg/ml) coated 96-well round bottom plates at 200 μl/well at 10^6 cells/ml with soluble anti-CD28 (1μg/ml) and incubated at 37° C for 3 days. Cells were then washed and rested in complete medium for 1 day, and aliquots of 2×10^3 cells/100 μl/well were plated and incubated for 2 days with varying concentration of IL-2 variants. [^{3}H]-thymidine was added, and 16 hours later cells were harvested and [^{3}H]-thymidine incorporation determined by liquid scintillation.

CFSE Dilution and EdU Proliferation assays

Freshly isolated pre-activated $CD8^+$ T cells (20 x 10^6/ml) were labeled with 2.5 μM CFSE (Molecular Probes) in PBS at room temperature for 7 min, washed once with 100% FBS (2 ml/sample), and then twice in complete RPMI medium. 2 x 10^6/ml. CFSE labeled cells were cultured in the absence or presence of wild type IL-2, H9, H9-RET, H9-RETR, or IL-2 plus H9-RETR. Cell proliferation was assessed by flow cytometric analysis of CFSE dilution at indicated time-points. For EdU proliferation assays, $CD8^+$ T

cells were pre-activated with anti-CD3 + anti-CD28 and cultured as above. 16 h before harvesting, 10 mM EdU was added, cells were stained for surface antigens as indicated, and then for intracellular EdU according to the manufacturer's protocol (BD Biosciences). Cell proliferation was assessed by flow cytometry.

Gene Expression Analysis by RT-PCR

Total RNA was isolated using RNeasy Plus mini kit (Qiagen) and 200 ng was used together with oligo dT (Invitrogen) and the Omniscript reverse transcription kit (Quiagen) to synthesize cDNA. Quantitative RT-PCR was performed with an ABI 7900 HD Sequence Detection System. RT primers and probes were from Applied Biosystems. Expression was normalized to *RPL7*.